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14. ABSTRACT

The hypothesis underlying this synergistic partnership award was that immunotherapy of epithelial ovarian carcinoma (EOC) with chimeric antigen receptor (CAR)-expressing T-cells would be potentiated if simultaneous targeting of tumor cells and tumor-associated macrophages (TAM) was achieved. To test this hypothesis, we initially engineered T-cells to express CARs with specificity for MUC1 (expressed by tumor cells) and CSF-1R (expressed both by tumor cells and TAM). *In-vitro* experiments demonstrated some efficacy of this therapeutic strategy. To test efficacy *in-vivo*, we used a previously established (EOC-like) tumor model based upon MDA-MB-435 cells, engineered to co-express MUC1 and CSF-1R. However, no significant therapeutic activity was observed in this model. As per an agreed revised statement of work, a CAR termed T1E28z was constructed which targets T-cells against several ErbB receptor dimers that are upregulated in EOC. Liposomal clodronate was used to achieve depletion of TAM. We showed that T1E28z-transduced T-cells were effective in mediating the killing of both autologus patient-derived tumor cell cultures and EOC cell lines (IGROV-1 and SKOV-3). Next, xenograft EOC models were established using luciferase-expressing SKOV-3 tumor cells. Using bioluminescence imaging (BLI), we demonstrated that T1E28z^{+} T-cells mediated the regression of established SKOV-3 tumors in SCID Beige mice. Whilst highly efficient depletion of TAM has been achieved using liposomal clodronate, this did not influence anti-tumor activity in this model. However, potency of immunotherapy was enhanced by repeated administration of T1E28z-engineered T-cells. T-cell activity was imaged in this study by co-expression of renilla luciferase in these cells. This analysis revealed that T-cells undergo progressive decline in tumor-bearing mice, providing a strong rationale for repeated administration to achieve maximum efficacy.

Table of Contents

Page

1. Introduction Ovarian cancer remains the most lethal of the gynecologic malignancies, largely owing to its propensity for clinical silence, late presentation and progressive evolution of chemoresistance. In 2012, an estimated 15,500 American women have already or will die of this disease, most commonly of the Epithelial Ovarian Carcinoma (EOC) subtype (1). Despite recent advances in management, this number represents almost 70% of predicted disease incidence in the same year (1). Consequently, the need for innovative therapeutic approaches for this devastating tumor is clearly evident.

The objective of the proposed research was to genetically engineer human T-lymphocytes using CAR technology, thereby enabling them to kill epithelial ovarian carcinoma in a safe and effective manner. To target tumor cells, we tested CARs that engage either the MUC1 mucin (2, 3) or the extended ErbB family of receptor tyrosine kinases (4). Macrophage infiltration has been linked with poor prognosis in EOC in several studies (5-8). Consequently, therapeutic interventions that reduce TAM infiltration have been tested for therapeutic activity in EOC (9). We hypothesized that efficacy of CAR-based immunotherapy would be potentiated by the simultaneous depletion of tumor-associated macrophages. Two approaches were used to test this. Initially, we used a CAR that targets colony-stimulating factor-1 receptor (CSF-1R), which is expressed by TAM in EOC (10). Alternatively, experiments were performed following treatment with liposome encapsulated clodronate, which is highly effective at depleting macrophages *in-vivo* (11).

2. Body

Below, we have presented our research activity over the duration of the project (July 1st 2011 to October 31st 2012). Data are described against the agreed Statement of Work. Where relevant, reference is made to previously submitted annual reports.

Task 1 – Finalize Ethical Approval to obtain blood and ascites from patients with EOC, enabling experimental work to be conducted in both research facilities (*SGM*).

Target	Deliver by initiation of funding.
Status	Achieved.

Task 2 – Obtain a Project License from the United Kingdom Home Office to provide legal authority to conduct controlled procedures on mice, as specified in the accompanying proposal (*SGM*).

Target	Deliver by 6 months.
Status	Achieved.

Task 3 - Introduce Chimeric Antigen Receptors (CAR) to T-cells derived from patients with EOC (*JM*).
Peripheral blood mononuclear cells (PBMC) were activated using CD3+CD28 expander beads. It was originally planned to deliver four CARs to separate T-cell populations using the SFG retroviral vector and retronectin-coated tissue culture dishes: (i) HOX – targets MUC1 and contains a fused CD28+OX40+CD3ζ endodomain (2, 3) (ii) CSF28z – targets CSF-1R and contains a fused CD28+CD3ζ endodomain; (iii) HDFTr - targets MUC1 and contains a truncated (inactive) endodomain (2, 3); (iv) CSFTr targets CSF-1R and contains a truncated (inactive) endodomain. However, we found that neither of the original targeting strategies proved to be effective, as described fully in Annual report year 2. In brief, we showed that both CARs were functionally

active when tested *in-vitro* by co-culture of engineered T-cells with tumor monolayers that expressed cognate antigen. However, adoptive immunotherapy with this combination of T-cells was ineffective *in-vivo,* when tested in mice bearing an established aggressive MUC1-expressing tumor. As previously reported, an alternative approach using CARs targeting the ErbB family of recepors was developed and a revision to the original statement of work was agreed.

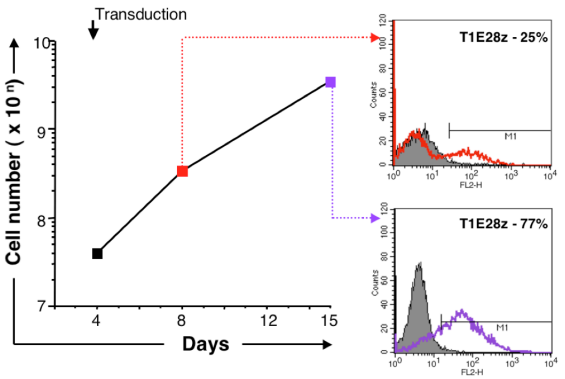

Figure 1. Expansion of T4-engineered T-cells. Healthy donor T-cells were transduced with the SFG T4 vector in which the T1E28z chimeric antigen receptor is co-expressed with the 4αβ chimeric cytokine receptor. After gene transfer, cells are cultured in IL-4 which provides a selective advantage to the gene-modified T-cells. Note the exponential T-cell expansion achieved, which is accompanied by enrichment of the gene-modified cells within the culture.

These CARs are:

i) T1E28z, which targets ErbB dimers and contains a fused CD28 + CD3ζ endodomain (4);

ii) T1NA – a control CAR that also targets ErbB dimers but contains a truncated (inactive) endodomain (4);

iii) T4- targets ErbB dimers (T1E28z) but also contains the 4αβ chimeric cytokine receptor (12). In 4αβ, the ectodomain of IL-4Rα has been coupled to the signaling domain of IL-2/15Rβ. Consequently, IL-4 (which is a weak T-cell mitogen) delivers a potent growth signal to 4αβ-expressing T-cells. Co-expression of T1E28z and 4αβ was achieved in the T4 vector with the ue of an intervening *Thosea Asigna* (T2A) peptide (4). Use of the IL-4/ 4αβ system is convenient since it permits the selective expansion and enrichment of T1E28z-engineered T-cells using IL-4. An example of this, using T-cells isolated from a healthy donor, is shown in **Figure 1**.

iv) P4 (P28z + 4αβ) – this combination comprises the P28z CAR (13; targeted against an irrelevant antigen, prostate membrane specific antigen (PMSA)) and co-expressed with 4αβ using an intervening T2A peptide.

v) T1E-Ren – in which the T1E28z CAR is co-expressed with luciferase derived from *renilla reniformis* (ren-luc), using the T2A system.

vi) T1NA-Ren – in which the T1NA control CAR is co-expressed with ren-luc, also using the T2A system.

Target	Deliver first gene transfer experiment within 8 months.
Status	Achieved and described in Annual report year 1.

Task 4 – Separate EOC tumor cells and tumor-associated macrophages from ascites by flow sorting (*SGM*). Ascites will be removed from a total of 25 patients with EOC with informed consent over the duration of the study (3 years).

Target	Demonstrate feasibility by month 6.
Status	Achieved and described in Annual report year 1.

Revised Task 5 - Co-cultivate engineered T-cells with tumor cells/ ascites (*JM*). T-cells will be mixed at 1:1 ratio in the following combinations: (i) T1E28z (test); (ii) T1NA (truncated control CAR). We will use the 4αβ chimeric cytokine receptor to facilitate expansion and enrichment of gene-modified T-cells. The effect of macrophage depletion cannot reliably be tested *in-vitro* and will be explored *in-vivo*.

Target Complete by month 24.

Status Achieved and described in part in Annual report year 2.

In our previous report, (Figure 3 – year 2 report), data was presented to show the superior EOC tumor targeting properties of IL-4-enriched T4-engineered T-cells, compared to control cells that expressed the P4 vector. In brief, we reported that patient-derived $T4^+$ T-cells elicit complete destruction of monolayers derived from (i) autologous tumor ascites; (ii) IGROV-1 cells and (iii) SKOV-3 cells. This was accompanied by production of several CTL related cytokines by activated $T4^+$ T-cells, including IL-2 and IFN-γ.

Progress since last report: Over the past 15 months, work has focussed on the establishment of autologous co-cultivation experiments in which engineered patient T-cells were co-cultivated with autologous tumor cells. When cultured as monolayers in serum-containing medium, we found that EOC tumor cell cultures are commonly overgrown by fibroblasts, with loss of $EpCAM^+$ tumor cells. Consequently, in order to maintain $EpCAM^+$ tumor cells in culture for longer periods of time, we established an alternative culture system whereby cells are cultured in ultralow serum medium that contains EGF, bFGF and insulin. To minimize differentiation during this period, cells are propagated in low adherence plates. Under these conditions, tumor cells form spheres of approximately 50 – 200 cells which are referred to as tumorspheres (**Figure 2**). By flow cytometry, we demonstrated that such tumorspheres are enriched for the presence of $EpCAM^+$ tumor cells and express ErbB receptors (**Figure 2A**). While tumorspheres were maintained in culture, T-cells derived from the same patients were engineered in parallel to express T4 or control CARs. When autologous tumorsphere/ $T4^+$ T-cell co-cultures were established, we consistently observed that $T4^+$ but not control T-cells eliminated tumorspheres rapidly from the cultures (**Figure 2B-D**), accompanied by production of interferon-γ (**Figure 2C**). Out of 12 patients tested, all patient derived $T4^+$ T-cells were capable of destroying autologous tumor cells.

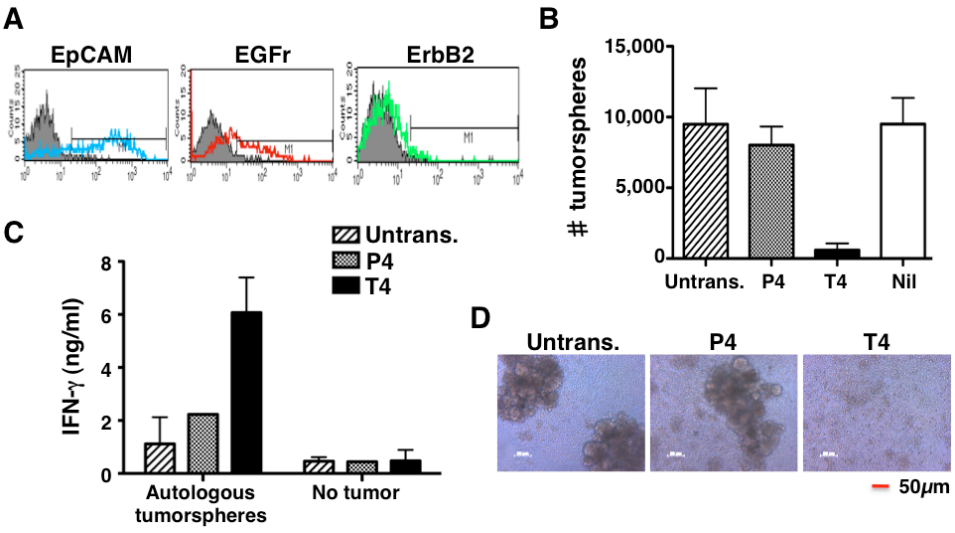

Figure 2: Anti-tumor activity of $T4^+$ T-cells against primary EOC tumor-spheres. (**A**) Disaggregated primary tumors or ascites cells were cultured in low serum-containing medium containing EGF, bFGF and insulin for 15 days, in ultra low adherence cell culture plates. Sphere cells were then analyzed for expression of EpCAM, EGFr or ErbB2 by flow cytometry (open histograms; filled histograms show isotype control staining). (**B**) T-cells and tumorspheres (average size 100 tumor cells) were co-cultured at a 100:1 ratio for 24 hours. Residual tumorspheres were enumerated by trypan exclusion (mean ± SEM, n=4 (Untrans, T4, Nil) or 2 (P4). (**C**) Interferon (IFN)-γ production was analyzed in supernatants harvested from T-cell tumorsphere co-cultures; (**D**) Representative microscopic image obtained after 24 hours.

Task 6 – Generate ffLUC-expressing SKOV-3 cells by retroviral-mediated gene transfer (*JM*).

Target Deliver by month 12.

Status Achieved and described in Annual report year 2.

Task 7 – Establish tumorigenicity of ffLUC-SKOV-3 and ffLUC-IGROV-1 following intraperitoneal injection in SCID Beige mice, as measured clinically and using bioluminescence imaging (*SGM*). To permit administration of 3 tumor cell doses to groups of 5 mice, 30 mice will be required for both models.

Target Deliver by month 18.

Status Achieved and described in Annual report year 2.

Task 8 – Generate sufficient CAR/ rrLUC$^+$ T-cells to treat tumor-bearing mice by intraperitoneal injection (*JM*). From 50ml blood, we can generally isolate 5 x 10^7 PBMC. Will need to achieve approximately 10-fold T-cell expansion *in-vitro* to allow the administration of 2 x 10^7 T-cells per mouse (described in Task 9). This level of T-cell expansion is generally achievable using CD3+CD28 expander beads in 7 – 10 days.

Target Deliver by month 20.

Status Achieved and described in Annual report year 2.

Task 9 – Monitor tumor-progression in mice following T-cell therapy, using dual bioluminescence imaging (*SGM*). Five groups of mice will be used in these experiments:
Group 1 receive liposomal clodronate + T1E-28z T-cells.
Group 2 receive liposomal clodronate + T1NA T-cells.
Group 3 receive T1E-28z T-cells alone.
Group 4 receive T1NA T-cells alone.
Group 5 receive PBS.
These experiments will require a total of 140 mice. This number will allow 2 experiments per tumor model, each requiring 35 mice as follows: Five groups of 7 mice which will be treated as indicated above. Numbers have been calculated to permit meaningful statistical analysis while allowing for principles of reduction, refinement and replacement. In all cases, animals will be sacrificed if any symptoms develop, or in the event of progressive tumor growth (indicated by increasing bioluminescence signal intensity). If tumor rejection occurs, animals will be maintained in the facility for their natural lifespan.

Target Deliver first therapeutic experiment by month 24 (extended deadline until month 36)

Status Achieved

In the previous report (Year 2 - Figure 6) data was presented demonstrating that depletion of TAMs using liposomal clodronate alone exerted no significant effect on tumor progression in mice bearing EOC xenografts, despite complete depletion of intraperitoneal (F4/80$^+$) macrophages in these mice. Combination therapy of liposomal clodronate with T-cells expressing the T1E28z derived CAR "T4" also demonstrated no significant improvement over T4 therapy alone (Year 2- Figure 7F). This experiment did however reveal that T4$^+$ T-cells delivered to established SKOV3 tumor xenografts resulted in a marked, albeit transient tumor regression and significantly delayed tumor progression thereafter (Year 2-Figure 7F), justifying the revised approach whereby ErbB dimers are targeted. A single dose of T4$^+$ T-cells led to a rapid reduction in tumor burden within one week of T-cell delivery. Although tumor regrowth occurred thereafter, this was significantly reduced compared to both untreated mice or those treated with control P4$^+$ T-cells.

Progress since last report: Several experiments have been performed in order achieve this goal and to refine immunotherapy using T4 engineered T-cells.

1) Based on these preliminary findings, a second experiment was performed to confirm therapeutic efficacy of $T4^+$ T-cells against EOC xenografts. Animals were injected with 1 x 10^6 firefly luciferase-(ff-luc) expressing SKOV3 cells and treated once tumors were established in the peritoneal cavity. Mice were treated with either a single dose of 10 x 10^6 T-cells on day 18 as was performed in the initial experiment described in the Year 2 report, or with a second dose on Day 25 in an attempt to enhance tumor destruction and further delay tumor regrowth. Consistent with previous findings, a single dose of $T4^+$ T-cells resulted in rapid tumor regression within one week of delivery (**Figure 3A**). Again, consistent wih previous data, tumor burden gradually increased after that but was significantly lower than that in either untreated (PBS) or control ($P4^+$ T-cell treated) mice at the experimental endpoint on Day 60 (**Figure 3A** and **Figure 3C**). Animals treated with a second dose of T-cells demonstrated further tumor regression over the treatment period although tumors again progressed within 2 weeks of the final dose (**Figure 3A**). Treatment was well tolerated; whilst weight loss was observed in those mice receiving $T4^+$ T-cells in the week following the initial dose (**Figure 3B**) this was temporary and regained by the next week. Interestingly, weight was regained despite a second dose of $T4^+$ T-cells on Day 25 suggesting that repeated doses are well tolerated in this model.

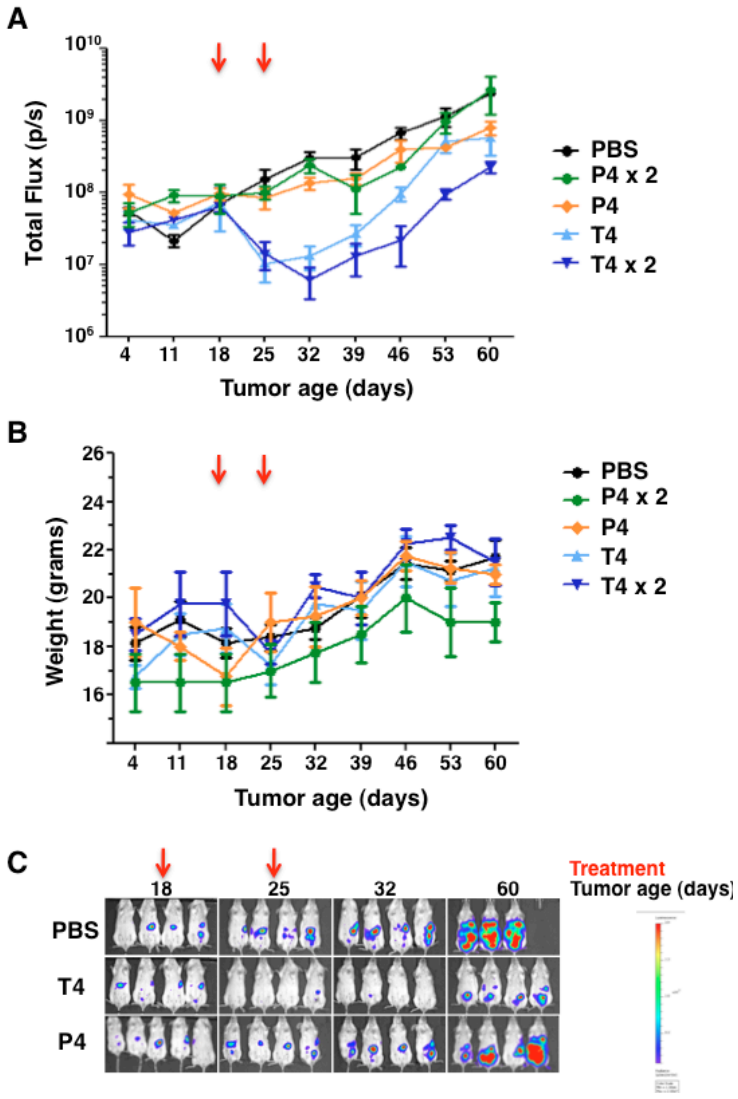

Figure 3: Comparison of single versus repeated administration of T4 immunotherapy. (**A**) SCID Beige mice were injected intraperitoneally (IP) with 1 x 10^6 SKOV3 cells that express firefly luciferase (SKOV-luc). After 18 days, mice bearing established IP tumors were treated with either a single IP dose of 10 x 10^6 $T4^+$ T-cells or 10 x 10^6 $P4^+$ T-cells. Where indicated, a second similar dose of T4 immunotherapy was administered on day 25 (indicated by arrows). Tumor burden was monitored weekly by bioluminescent imaging (BLI). The graph shows the mean +/- SD BLI emission from each of the indicated groups (n=4) over a timecourse of 60 days. (**B**) Mean body weight +/- SD of each of the treatment groups. Body weight, along with other symptoms, was used to assess toxicity of treatment. (**C**) Bioluminescence images of tumor burden at the indicated representative timepoints. Mice received 2 treatments with $T4^+$ T-cells, $P4^+$ T-cells or PBS. Treatment timepoints are indicated by the arrows.

2) In order to image T-cells *in-vivo*, the ren-luc gene was co-expressed with the T1E28z and T1NA CAR constructs. Ultimately, we hoped that this system would permit dual imaging of both tumor cells and T-cells following the administration of D-luciferin (ff-luc) and coelenterazine substrates (ren-luc) respectively. Function of these constructs was first tested *in-vitro*, following the transduction of T-cells from a healthy donor (**Figure 4A**). **Figure 4B** shows that luciferase activity of both constructs is satisfactory. Furthermore, co-expression of renilla luciferase does not adversely affect the ability of the T1E28z CAR to mediate the destruction of ErbB$^+$ EOC cell monolayers (**Figure 4C**).

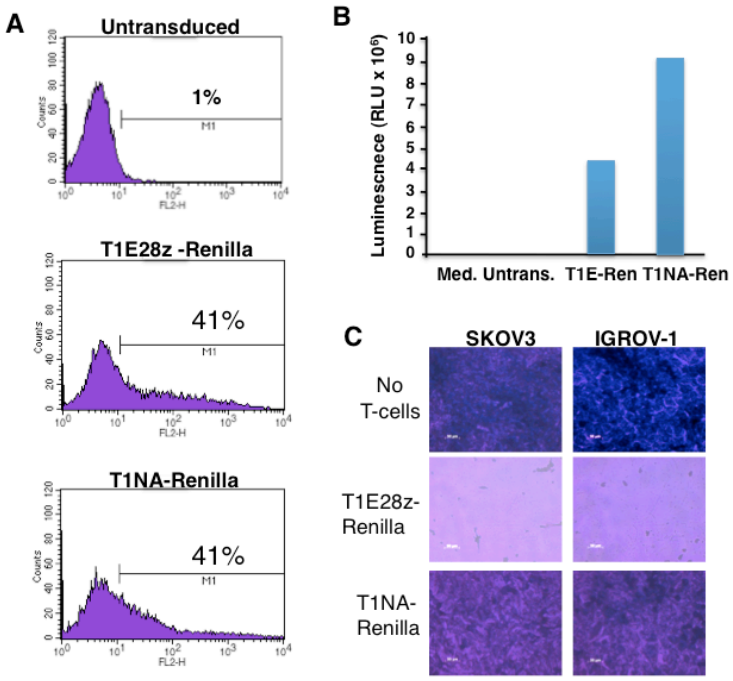

Figure 4: Validation of renilla-containing retroviral vectors. (**A**) Human T-cells were activated with CD3 + CD28-coated paramagnetic beads and were transduced with the T1E-Renilla or T1NA-Renilla retroviral vectors. Expression of the T1E28z CAR was detected by flow cytometry after incubation with an anti-EGF antibody that detects that T1E targeting peptide. Markers have been set using untransduced T-cells. (**B**) Activity of renilla luciferase was tested in transduced T-cells using an *in-vitro* Renilla-GLO assay, 10 minutes after addition of coelenterazine substrate. Data are presented as relative light units (RLU), compared to untrans(duced) cells and med(ium) only controls. (**C**) Transduced T-cells (1 x 10^6 cells) were co-cultivated in a 24 well dish with a confluent monolayer of the indicated tumor cells. After 24 hours, the residual monolayer was fixed and then stained with crystal violet.

3) In parallel with 2), an SFG retroviral construct was also engineered in which ren-luc was co-expressed with green fluorescent protein (GFP). This was used to help to set up techniques required for bioluminescence imaging of ren-luc-expressing T-cells in mice. To determine the best route of delivery of the ren-luc substrate (coelenterazine), a pilot study was performed in which T-cells that co-express ren-luc and GFP were administered IP to tumor-free mice. Coelenterazine was then administered using either the intraperitoneal (IP) or intravenous (IV) routes. **Figure 5A** shows that ren-luc-expressing T-cells were easily detectable when coelenterazine was administered IP. Highest signal intensity was evident 1 hour after T-cell injection, followed by progressive decline in signal intensity over the ensuing 48 hours. In contrast, imaging was markedly less sensitive following IV injection of coelenterazine. Using this route of delivery, ren-luc-expressing T-cells could only be visualised when the sensitivity of detection was lowered to its limit (**Figure 5B**, lower panel). Having determined that IP delivery was preferable to IV delivery, a pilot dual imaging experiment was performed. Five animals were all injected IP with SKOV-3 tumor cells that expressed ff-luc and thus were amenable to bioluminescence imaging after the administration of d-luciferin. Four days later, 2 mice received an IP injection of T-cells that co-express ren-luc and GFP while the remaining 3 mice were injected with untransduced T-cells. **Figure 5C** shows a bioluminescence imaging study performed on these mice after the co-administration of both luciferin substrates. Animals were scanned at 6 wavelengths covering the full range of the visible light spectrum in an effort to distinguish between light emitted by ren-luc$^+$ T-cells and ff-luc$^+$ tumor cells. However, light emission was consistently greater in mice that had received ren-luc$^+$ T-cells at all emission wavelenghts tested. We confirmed that tumor burden was equivalent in all five mice by performing an imaging study on the following day after administration of d-luciferin alone (**Figure 5D**). These data indicate that luminescence emission from ren-luc T-cells cannot be distinguished from that released by ff-luc$^+$ tumor cells. Simultaneous dual imaging is therefore not

possible and, in future experiments, BLI will have to be performed on separate days to detect either tumor or T-cells.

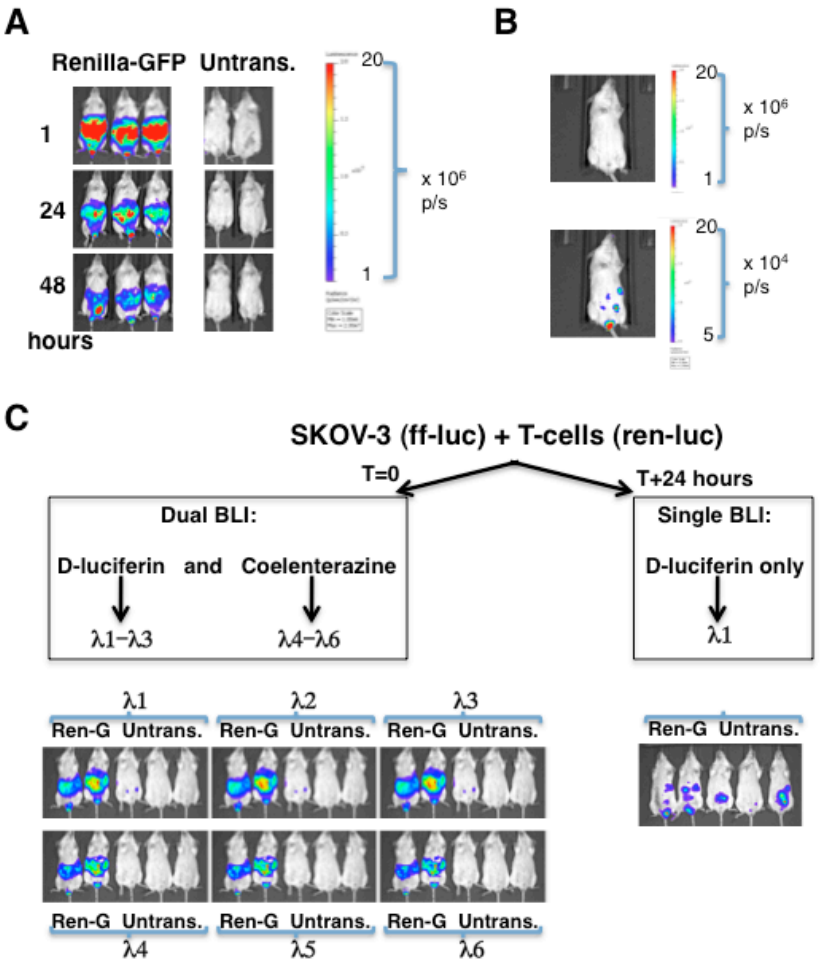

Figure 5: Bioluminescence imaging of T-cells that express renilla luciferase. (**A**) Human T-cells were engineered to co-express renilla luciferase (ren-luc) and GFP using the SFG retroviral vector and 10^7 cells were adoptively transferred to 3 SCID Beige mice by IP injection. Two control mice received a similar number of untrans(duced) T-cells. Imaging was performed at the indicated intervals after IP administration of colelenterazine. (**B**) Images obtained from a mice treated with ren-luc-T-cells as described in **A** and imaged following IV injection of coelenterazine. The sensitivity of the imaging performed in the upper panel is similar to that shown in **A** while sensitivity is 2 logs greater in the lower panel. (**C**) Pilot study of dual bioluminescence imaging. Five mice were injected with 2 x 10^6 SKOV-3 tumor cells that express firefly luciferase. Four days later, two mice received 10^7 ren-luc-expressing T-cells IP while 3 control mice received a similar number of untransduced cells. Bioluminescence imaging was performed after co-administration of d-luciferin and coelenterazine to detect (ff-luc) tumor cells and (ren-luc) T-cells simultaneously. Images were captured using 6 different wavelengths (λ1–λ6) to cover the whole spectrum of emission from both ff-luc and ren-luc. Individual boxes depict images taken at each wavelength. A single BLI study was performed 24 hours later following administration of d-luciferin only and confirms that tumor burden was equivalent in all animals.

4) Next, *in-vivo* studies were undertaken in order to test expression of functional renilla luciferase by T-cells following transduction with the T1E-Ren and TINA-Ren vectors, in which T1E28z or T1NA CARs are co-expressed with ren-luc. Anti-tumor efficacy of these T-cells was also tested by injection into mice bearing advanced SKOV3 xenografts that express ff-luc. **Figure 6A** demonstrates that transduced T-cells were detected for up to 9 days after intraperitoneal delivery. However, luminescence declined progressively over this timeframe, suggesting T-cell death, and was similar in animals that received T1E-Ren or control T1NA-Ren engineered T-cells. There was no effect on tumor burden following a single dose of T1E-Ren T-cells (**Figure 6B**). Two possible explanations may account for this finding: i) Tumors were treated at a very advanced stage (32 days) where previous experiments showing efficacy using $T4^+$ T-cells have started treatment at day 18 (**Figure 3A**); ii) Only 2 x 10^6 transduced cells were used in this experiment.

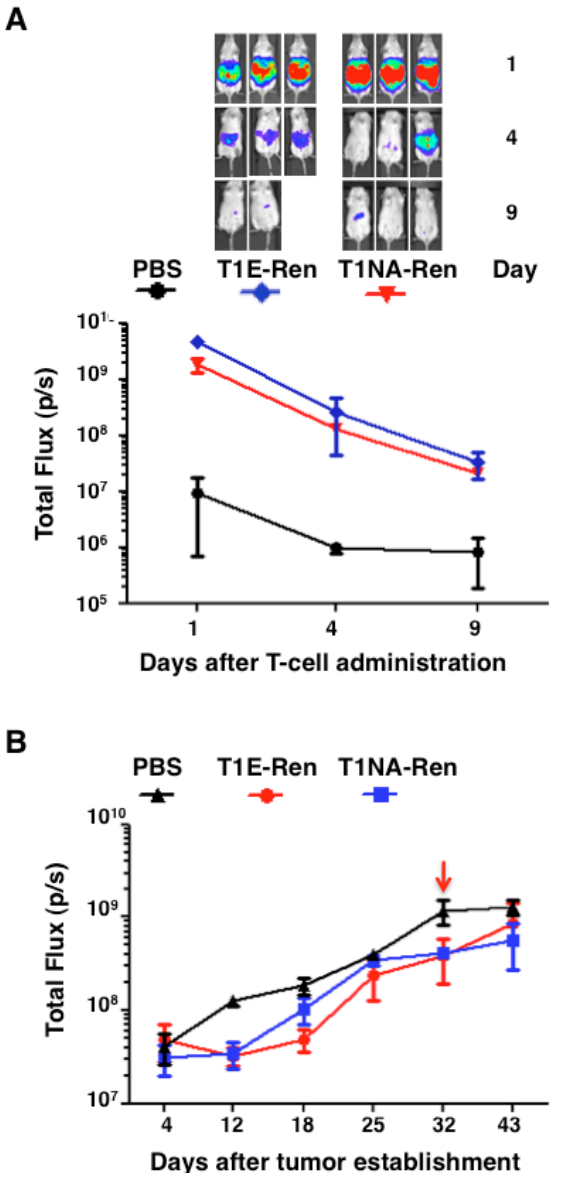

Figure 6: Bioluminescence imaging of CAR-engineered T-cells. (**A**) Human T-cells were transduced with the T1E-Ren or T1NA-Ren vectors. A total of 2 x 10^6 gene-modified cells were transferred IP into 3 mice each, making comparison with 3 mice that received PBS alone. All animals had advanced (32 day) intraperitoneal SKOV-3 ff-luc tumors. Upper panels shows light emission from groups of 3 mice at indicated timepoints, following IP administration of coelenterazine to image T-cells that express T1E-Ren (left) or T1NA-Ren (right). In the graph shown below, these data are expressed as mean ± SD for each time point. (**B**) Bioluminescence imaging of ff-luc-expressing SKOV-3 tumor was performed before and after T-cell administration by IP injection on day 32 (indicated by the arrow). In the graph, tumor light emission is presented as the mean ± SD. Note that one mouse in the T1E-Ren group was culled 4 days after T-cell transfer (eg day 36 of tumor growth) due to poor health.

Task 10 – Engineer vector to co-express CAR with human sodium iodide symporter (hNIS) using 2A cleavage system and deliver/ validate expression in human T-cells (*JM*).

Target Deliver by month 24 (extended deadline until month 36)

Status Achieved in part

The hNIS gene has been successfully inserted into the T4 vector (T1E28z + 4αβ) thereby generating a tricistronic vector named TiN-4. In parallel, a control vector has been produced in which the P4 vector (P28z + 4αβ) has been modified similarly. The structure of these vectors is shown schematically in **Figure 7A**. Expression of the transgenes encoded by TiN-4 is shown in **Figure 7B** (T1E28z) and **Figure 7C** (4αβ). Similarly, expression of P28z and 4αβ has been validated for the control PiN-4 vector (data not shown). We have been unable to establish a flow cytometry assay to detect hNIS in TiN-4, PiN-4 or a range of other cell types. Consequently, a western blotting assay has recently been developed for this purpose and has been used to validate hNIS expression in PiN-4 engineered PG13 cells and human T-cells (**Figure 7D**). Function of hNIS

has been confirmed in these cells by the demonstration of specific uptake of ^{99m}Tc-pertechnetate in a manner that is abrogated by the hNIS inhibitor, perchlorate (**Figure 7E**).

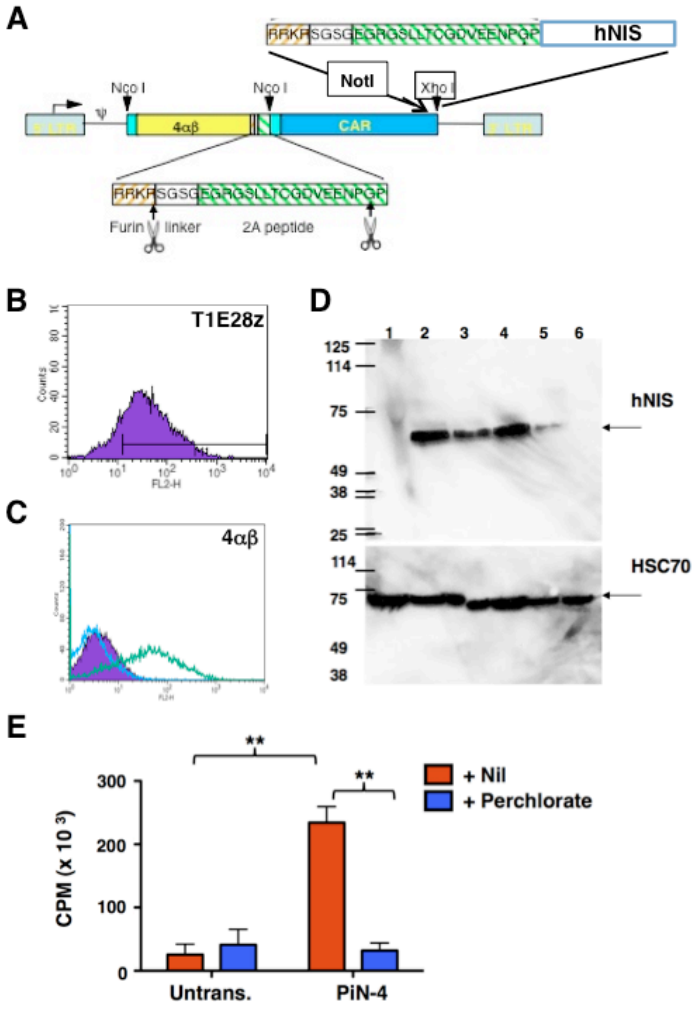

Figure 7: The TiN-4 and PiN-4 (control) vectors. (**A**) Schematic diagram of the TiN-4 and PiN-4 constructs. Stoichiometric co-expression of all three transgenes is achieved with the use of two intervening *Thosea Asigna* peptides, each placed dwonstream of a furin cleavage site (RRKR). Expression of transgenes has been validated in PG13 retroviral packaging cells. Expression of T1E28z (**B**) was detected by flow cytometry after incubation with an anti-EGF antibody. The indicated marker was set to include 99% of background reactivity in unmodified PG13 cells. (**C**) Expression of 4αβ was detected in TiN-4-engineered PG13 cells using anti-human IL-4 receptor-α antibody (open histogram – green line). Staining of unmodified PG13 cells with this reagent is shown as the open blue histogram while staining of TiN-4$^+$ PG13 cells with secondary antibody alone is indicated by the filled histogram. (**D**) Repeated efforts to detect hNIS by flow cytometry have been unsuccessful. However, expression of the hNIS protein has recently been confirmed in cells transduced with a matched control vector in which a PSMA CAR (P28z (13)) is co-expressed in the same manner with 4αβ and hNIS (named PiN-4). Lanes in the western blot show PiN-4 engineered PG13 cells (lane 4) and T-cells (lane 5). Lanes 1 and 6 show untransduced PG13 and T-cells respectively. Loading was controlled by probing with anti-HSC70. (**E**) Function of hNIS in PiN-4 engineered T-cells (5 x 10^5 cells) was confirmed by specific uptake of ^{99m}Tc-pertechnetate in a manner that is abrogated by perchlorate block. Counts were quantified using a gamma counter and are expressed as mean ± SD of triplicate results.

Task 14 – Establish primary EOC ascites-derived tumor cells in SCID Beige mice (+/- stem cell enrichment *ex-vivo*; *SGM*). We anticipate the use of 60 SCID Beige mice for these studies to facilitate dosing/ optimization.

Target Deliver by month 30 (extended deadline until month 40)

Status Experimental work initiated

Previously in the Year 2 report we described the purification of patient-derived tumor cells from ascites and tumor tissue using antibody markers associated with tumor cell 'stemness' (Year 2 report- Figure 8). These included CD117, CD133 and ABCB1. Tumor cells enriched for stemness and growing in spheroids in culture, another stem-like property, were injected subcutaneously into SCID-beige mice and monitored for tumor growth over a 10 week period. No tumors were palpable within this timeframe and mice then had to be culled under the conditions of our Home Office licence. Attempts to establish primary tumors in mice are ongoing.

Task 15 – Generate sufficient engineered T-cells that co-express CAR + hNIS for testing of therapeutic efficacy in mice bearing primary tumor xenografts (*JM*).

Target Deliver by month 32

Status To be completed once TiN-4 fully validated.

Task 16 – Monitor tumor-bearing mice following T-cell therapy using labeling of T cells and tumor cells and bioluminescence as well as SPECT/CY/PET imaging to obtain high resolution images of tumor deposits and T cell infiltration (*SGM*). Animals will be sacrificed as specified in Task 9. If animals reject tumor, they will be maintained in the facility for their natural lifespan (n=60 mice).

Target Deliver by month 36

Status This work will be initiated under the direction of the partnering PI in the final year of the revised and extended project.

3. Key Research Accomplishments

- Several relevant chimeric antigen receptors have been engineered, cloned and used to generate viral vector.
- Feasibility of transduction of patient T-cells has been repeatedly demonstrated, with efficiency even in patients with 'bulky' ovarian cancer.
- Use of the model based upon the MDA-MB-435 tumor cell line, allowing testing of our hypothesis with the preferred target pair (MUC1 and CSF-1R) has shown that this dual targeting approach is ineffective.
- By contrast, experimental data gathered using the ErbB-specific T1E28z CAR indicate that it achieves significant activity against EOC, both *in-vitro* and in SCID Beige mice bearing established tumor xenografts.
- Data suggests that tumor regrowth occurs within 2 weeks of T-cell delivery, consistent with the observation that transduced T-cells persist for approximately 9 days following delivery *in-vivo*. A second dose of ErbB-specific T1E28z CAR leads to enhanced activity against EOC in mice bearing established xenografts, suggesting that T-cell persistence may be a limiting factor in the achievement of sustained tumor response.
- Our results suggest that depletion of TAMs in the *in-vivo* SKOV-3 EOC model using clodronate does not have a beneficial effect. This finding contrasts with the accepted dogma concerning the deleterious role of TAMs in ovarian cancer.
- Renilla luciferase and hNIS imaging genes have been engineered into CAR-containing retroviral constructs and validated.
- Renilla luciferase-expressing T-cells can be imaged up to 9 days after delivery *in-vivo* using BLI. However, it is not possible to perform dual BLI of both tumor and T-cells due to similar emission spectrums of both firefly and renilla luciferase.
- All necessary approval has been granted for the development of positron emission tomography (PET) imaging of T-cells using hNIS. This work will be continued by the partnering PI in the final year of the project.
- Systems to identify and purify putative EOC stem cells have been put in place. Injection of these cells subcutaneously into SCID-Beige mice is yet to result in tumor growth.

4. Reportable Outcomes

1. Brewig N, Parente-Pereira AC, Maher J, Ghaem-Maghami S (2010) An in-vivo xenograft model to study simultaneous targeting of cancer cells and immunosuppressive tumour-infiltrating myeloid cells. British Society of Immunology (2010). **Immunology** 131 S1 Abstract 589.
2. Parente-Pereira AC, Brewig N, Ghaem-Maghami S, Maher J (2010) Immunotherapy of epithelial ovarian cancer using CAR engrafted T-cells: in vitro development. British Society of Immunology (2010). **Immunology** 131 S1 Abstract 601.

3. Parente Pereira AC, Whilding L, Brewig N, Chatterjee J, Maher J, Ghaem-Maghami S (2012) Targeting the ErbB family using chimeric antigen receptor (CAR) T-cells in epithelial ovarian cancer. Presented at the British Socitey for Gynaecological Oncology meeting, London 2012.
4. Whilding L, Parente Pereira A, Chatterjee J, Maher J, Ghaem-Maghami S (2012) Targeting the ErbB family using chimeric antigen receptor (CAR) T-cells in epithelial ovarian cancer. Presented at the NCRI meeting, Liverpool, 2012. Abstract B25.

No full publications have been produced as yet. At this point, we anticipate the completion of two papers that will describe efficacy of T4 immunotherapy in models of EOC and imaging of ErbB-re-targeted cells in these models.

5. Conclusions

The data presented in this report demonstrate that T-cells from patients with epithelial ovarian cancer can be genetically targeted against ErbB receptor dimers that are aberrantly upregulated on tumor cells. Importantly, we have demonstrated efficacy against established tumor xenografts *in-vivo* and using matched patient T-cell/ tumor cell co-cultivations *in-vitro*. Data gathered to date would not support a clinical rationale for targeting of TAM and research now focuses on ErbB directed tumor cell killing.

Data to date shows that T4 transduced T-cells substantially reduce tumor burden within one week of delivery, but that tumor regrowth occurs in the following week. Imaging studies performed this year demonstrate a strong presence of transduced T-cells in the days following injection which then decline over 9 days, suggesting that T-cell death may be a limiting factor in the efficacy of ErbB re-targeted T-cells. In support of this, a second dose of engineered T-cells delivered a week after the first dose led to enhanced tumor regression. This suggests that tumor cells surviving the initial dose are not resistant to ErbB directed therapy and can be killed by repeated treatment. There is a possibility that T-cell longevity can be increased by exploitation of the IL-4-regulated growth system we have developed (12) to achieve selective support of ErbB re-targeted T-cells *in-vivo*, particularly in the tumor microenvironment. PET imaging performed over the next year will also offer a more sensitive and clinically relevant approach to monitoring T-cell longevity. In parallel, we will shortly gather clinical experience with T4 immunotherapy when we initiate first in man testing in patients with head and neck cancer. We anticipate that this study will provide critical information that may be applied to develop this therapeutic approach further in ovarian cancer.

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